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The Synergistic Antimicrobial Effects of Novel Bombinin and Bombinin H Peptides from the Skin Secretion of *Bombina orientalis*

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The nucleotide sequence of the cDNA encoding BHL-bombinin and bombinin HL precursor from the skin secretion of *Bombina orientalis*, has been deposited in the EMBL Nucleotide Sequence Database under the accession code: LT615078

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Abstract

Bombinin and bombinin H are two antimicrobial peptide (AMP) families initially discovered from the skin secretion of *Bombina* that share the same biosynthetic precursor-encoding cDNAs, but have different structures and physicochemical properties. Insight into their possible existing relationship lead us to perform the combination investigations into their anti-infectious activities. In this work, we report the molecular cloning and functional characterisation of two novel AMPs belonging to bombinin and bombinin H families from secretions of *Bombina orientalis*. Their mature peptides (BHL-bombinin and bombinin HL), coded by single open-reading frame (ORF), were chemically synthesised along with an analogue peptide that replaced L-leucine with D-leucine from the 2nd position of the N-terminus (bombinin HD). Circular dichroism analysis revealed that all of them displayed well-defined α-helical structures in membrane mimicking environments. Furthermore, BHL-bombinin displayed broad-spectrum bactericidal activities on a wide range of micro-organisms, while bombinin H only exhibited a mildly bacteriostatic effect on the Gram-positive bacteria *Staphylococcus aureus*. The combination potency of BHL-bombinin with either bombinin HL or bombinin HD showed the synergistic inhibition activities against *S. aureus* (FICI 0.375). A synergistic effect has also been observed between bombinin H and ampicillin, which was further systematically evaluated and confirmed by *in vitro* time-killing investigations. Haemolytic and cytotoxic examinations exhibited a highly synergistic selectivity and low cytotoxicity on mammalian cells of these three peptides. Taken together, the discovery of the potent synergistic effect of AMPs in a single biosynthetic precursor with superior functional selectivity provides a promising strategy to combat multidrug-resistant pathogens in clinical therapy.

Keyword**:** peptide; skin secretion; antimicrobial; synergism; selectivity

1. Introduction

Bombinin, one of the typical cationic AMPs, was firstly isolated from the skin secretion of the yellow-bellied toad *B. variegata* [1]. The nucleotide sequence analysis of bombinin-related peptides

prompted the existence of a class of structurally differentiated peptides, which were named as bombinin H [2,3]. Importantly, the presence of a subtle and inconspicuous single D-amino acid (D-alloisoleucine or D-leucine) at the second position from N-terminus of bombinin H, as a consequence of post-translational modification, was observed. This type of modification may contribute to the versatile antimicrobial mechanisms of frog skin peptides, and may be beneficial to the prevention of bacterial resistance [4-6]. However, since the initial discovery of bombinin, bombinin H and D-isoform bombinin H, research have been focused on the study of the individual peptide's antimicrobial property, instead of their synergistic potencies. Combined effects of bombinin peptides with conventional antibiotics, and their antimicrobial selectivity towards pathogens have very rarely been reported [7].

Here, we report the structural and functional characterisation of two novel, linear cationic α-helical AMPs, initially identified in a single ORF from the skin secretion of *Bombina orientalis*. These peptides belong to the bombinin and bombinin H families. The potent synergistic relationship of the novel bombinin and bombinin H peptides highlights the significance of combinational utility of AMPs in the treatment of infections caused by drug-resistant bacteria, this continues to provide researchers with novel approaches for prospective innovation in clinical studies.

2. Materials and Methods

2.1 Specimen preparation and secretion harvesting

Specimens of the oriental fire-bellied toad *B. orientalis* were obtained from a commercial supplier and raised in a specially designed vivarium until mature, over a period of 4 months. The skin secretions were collected and lyophilised as previously described [8]. Sampling of skin secretion was performed by Mei Zhou under UK Animal (Scientific Procedures) Act 1986, project license PPL 2694, issued by the Department of Health, Social Services and Public Safety, Northern Ireland. Procedures had been vetted by the IACUC of Queen's University, Belfast, and approved on 1 March 2011.

2.2 Molecular cloning of novel bombinin and bombinin H precursor-encoding cDNA from the skin secretion-derived cDNA library

A five-mg lyophilised secretion of *B. orientalis* was dissolved in 1 ml of mRNA protection buffer, the polyadenylated mRNA was obtained by using magnetic oligo-dT beads following the instructions of the manufacturer (Dynal Biotech, Wirral, UK), and subsequently reverse-transcribed. The cDNA was subjected to 3'-RACE PCR procedure to obtain the full-length prepro-bombinin and prepro-bombinin H nucleotide sequence using a SMART-RACE kit (Clontech, Oxford, UK) as described by the manufacturer. For 3′ RACE reaction, a nested universal primer (NUP) (supplied with the kit) and a degenerate sense primer were designed and performed as previously reported [9,10]. The 3′-RACE reactions were performed as previous description [11].

2.3 Identification and structural analysis of deduced mature peptides in the skin secretions

Another 5 mg of lyophilised secretion were dissolved in 1.0 ml of 0.05/99.95 (v/v) trifluoroacetic acid (TFA)/water and clarified by centrifugation. The rp-HPLC system was fitted with an analytical column (Phenomenex C-5, 0.46 × 25 cm and pheomenex C-18, 250×10mm), eluting with a linear gradient formed from trifluoroacetic acid (TFA) / dd water (0.05/99.95, v/v) to TFA / dd water / Acetonitrile) (0.05/19.95/80.0, v/v/v) in 240 min at 1 ml/min. The fractions were collected automatically at minute intervals and effluent absorbance was continuously monitored at λ 214 nm and λ 280 nm. Each reverse phase HPLC fraction was analysed with matrix-assisted, laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE mass spectrometer (Perseptive Biosystems, MA, USA) in positive detection mode using α-cyano-4-hydroxycinnamic acid as the matrix. Fractions containing peptides with molecular masses coincident with predicted mature peptides from 'shotgun' cloning were infused into the LCQ Fleet™ ion-trap electrospray mass spectrometer for analysis (Thermo Quest, San Jose, CA, USA).

2.4 Peptides synthesis and purification

The two-novel identified bombinin peptides and one single residue D-isomer analogue were synthesised by Tribute® Peptide Synthesizer (Protein Technologies, Inc., Tucson, USA) with solid-

phase Fmoc chemistry methodology and amide resin. Their molecular masses were analysed and confirmed by MALDI-TOF. Then, synthetic replicates were purified with rp-HPLC to obtain high-purity of synthetic peptides.

2.5 Circular dichroism (CD) spectroscopy

CD spectra between 190 nm and 250 nm were performed on a Jasco J-815 CD spectrometer (Jasco, Essex, UK). The machine units of millidegrees ellipticity were converted to mean residue molar ellipticity using the following equation (n: the number of peptide bonds; Ellipticity is the raw data from the instrument):

θ (deg.cm^2.dmol^{-1}) = Ellipticity (mdeg)*10^6 / Pathlength (mm)*[Peptide] (µM)*n

The spectra were recorded at 100 nm/min in ammonium acetate (10 mM) buffer or TFE (50%) solution. CD measurements were performed at 20 °C with 1 mm path length cuvette. An average of three scans were collected and automated analysed for each peptide. The final predicted percentage of secondary structure was calculated using the K2D3 CD spectra web server [12].

2.6 Antimicrobial activity and minimal biofilm eradication concentration (MBEC) assays

The minimal inhibitory concentrations (MICs) of the synthetic replicates of the AMPs were determined using quality control strains, the Gram-positive bacterium, *Staphylococcus aureus* (NCTC 10788), the Gram-negative bacteria *Escherichia coli* (NCTC 10418) and *Pseudomonas aeruginosa* (ATCC 27853), the yeast *Candida albicans* (NCPF 1467), and MRSA (Methicillin-resistant *Staphylococcus aureus*) (ATCC 12493). The reference strains of the microorganisms were initially incubated in Mueller-Hinton Broth (MHB) for 16–20 h, then the bacterial cultures were diluted to obtain 1×10^6 cfu/ml for the bacterial and the yeast culture to 5×10^5 cfu/ml. The samples were added to to obtain final concentrations from 1 to 512 mg/l. After 24 h incubation, the OD of each well was measured at 550 nm. The MIC value was measured as the minimal concentration of peptide with an OD identical with that of the negative controls [13]. Upon achieved the data from MIC assays, 10 µl of the medium from each well was taken and inoculated onto Mueller-Hinton agar (MHA) plates. After 24 h incubation, the minimum bactericidal concentrations (MBCs) and the minimum fungicidal

concentration (MFC) were obtained, which were defined as the lowest concentration of peptide from which no colonies could be subsequently grown.

The MBECs of the synthetic peptides were determined against *Staphylococcus aureus* and performed following a standard method as shown in manufacturer's instruction (Innovotech, UK). The MBEC™ P&G assay plate with specialised peg architecture that is designed for the formation of biofilm was used for anti-biofilm susceptibility tests. The procedure of inoculate and subculture were performed as described before. The inoculum plate was prepared by transferring 200 µl inoculum to the 96-well plate and kept in a 150-rpm moist orbital incubator for 72 h at 37 °C. After which, the lid with pegs of the inoculum plate was rinsed by PBS twice, seven replicates of a serial of two-fold diluted peptides (1–512 mg/l) along with the positive/negative controls were added to corresponding wells. After incubation at 37 °C for 24 h, the recovery plate was prepared by adding 200 µl recovery medium (MHB/neutralising agents 20/0.5 (v/v)) into each well. The lid from the inoculum plate was rinsed and the placed to the recovery plate. After sonication for 30 min, the recovery plate was measured at 550 nm. The minimal biofilm eradication concentration was determined as the lowest concentration with no microbiology growth was detected. Melittin (Sigma-Aldrich, UK), firstly isolated from honeybee (Apis mellifera) venom, was taken as positive controls in comparison [14,15]. Both antimicrobial and biofilm eradication assays were independently performed three times.

2.7 Kinetic time-killing assays

The kinetic time-killing assays were performed with different concentrations of peptides alone or with another checkerboard titration predicted synergistic agents. The concentration series of peptides alone or with the synergistic counterpart were added to 1.5 ml micro-centrifuge tubes, which were then inoculated with a log phase culture of the test organism as described in the previous section. During the incubation, 50 µl sample of each tube was removed from culture tubes at 0, 5, 10, 20, 30, 60 and 120 min intervals for single peptides or 0, 0.5, 1, 3, 6, and 24 h intervals for synergistic pairs. After diluted serially with PBS, 50 µl of diluted samples were inoculated on MHA plates and incubated at 37 °C for 24 h for colony counts. The synergistic effect was defined as equal or higher to 2 - $\log_{10}$ -

CFU/ml decrease in bacterial counts compared to the effect of the most active single constituent [16]. Curves were constructed by plotting the $\log_{10}$ of CFU/ml against time.

2.8 Haemolysis assay

Defibrinated horse erythrocytes (TCS Biosciences Ltd, Buckingham, UK) were prepared to produce a 4% (v/v) suspension of red blood cells in PBS by repeated washings with sterile PBS. A range of concentrations of synthetic peptides (1 to 512 mg/l) were incubated with red blood cell suspension samples (200 µl) at 37 °C for 120 min. After incubation, the suspensions of each sample were centrifuged to obtain the final lysis of red blood cells. OD measurements of supernatants were recorded at 550 nm. Negative controls were prepared by a 2% (v/v) suspension with PBS in equal volumes (0% haemolysis), while the positive controls employed consisting a 2% (v/v) suspension with 2% (v/v) of the non-ionic detergent, Triton X-100 (Sigma–Aldrich, UK) in PBS. HC_{50} was defined as the peptide concentration that caused 50 % haemolysis.

2.9 Cytotoxicity testing

The cytotoxicity of synthetic peptides on mammalian cells was examined using human microvessel endothelial cell (HMEC-1), which were cultured with MCDB 131 medium (Gibco, UK) supplemented with 10% FBS, 10 mM L-Glutamine, 10 ng/ml EGF and 1% penicillin streptomycin. 5^10^3 cells/well were seeded into 96-well plates. After 24 h incubation at 37 °C with 5% CO_2, 12 h serum-free starvation was performed, peptides with 10^{-9}–10^{-4} M concentrations were added for 24 h treatment prior to 10 µl MTT (5 mg/ml PBS) incubation for 4 h, the grow medium was removed followed by adding 100 µl of DMSO to dissolve the formazan crystals. The absorbance was measured at 570 nm. Data from this study were analysed by t-test using GraphPad Prism (version 5.01). A p value less than 0.05 was considered a significant difference. Negative and positive control treatments were carried out with culture medium and 1% Triton X-100, respectively. Data from this study were analysed by one-way ANOVA with Bonferroni's post-test.

2.10 Evaluation of combination effects of antimicrobial peptides

A two-dimensional checkerboard with 2-fold dilutions of each AMP was used for examining the combination effects with *S. aureus*. The dissolved samples of each peptide or antibiotic agent were diluted from 4×MIC to 1/16 ×MIC. The series of component A were added along the row of a 96-well plate, while the columns were filled with the diluted component B. Growth control wells containing only micro-organism medium and sterility control wells with only MHB medium were included. After the addition of a log-phase bacterial inoculum of 1×10^6 cfu/ml, plates were incubated at 37 °C for 24 h and then measured at λ 550 nm. The combination effects were examined by calculating the fractional inhibitory concentration index (FICI) of each combination as follows:

$$\frac{\text{MIC of component A, tested in combination}}{\text{MIC of component A, tested alone}} + \frac{\text{MIC of component B, tested in combination}}{\text{MIC of component B, tested alone}}$$

After the combination ratio of the two tested compounds was confirmed, lower concentration pairs were selected to determine the FICI with more accuracy. The profile of the combination was interpreted as synergistic for FICI≤0.5, additive for 0.5<FICI≤4.0, and antagonistic for FICI>4.0 [17,18].

For assessing the synergetic activity of bombinin and bombinin H against the growth of mammalian cell lines, both CalcuSyn software [19] and Jin's formula [20] were employed. Combination index (CI) plots were generated by using CalcuSyn software. A value of CI < 1 represents synergy. The following formula in Jin's formula was used: Q=Ea+b (Ea+Eb-Ea×Eb). Q is the combination index; Ea+b represents the cell proliferative inhibition rate of two AMPs; Ea and Eb represents the cell proliferative inhibition rate for individual peptide. After calculation, the results Q > 1.15 indicates synergy, and 0.85 < Q < 1.15 indicates an additive effect [19].

3. Results

3.1 Molecular cloning of skin secretion precursor cDNA encoding bombinin and bombinin H

The full-length biosynthetic precursor-encoding cDNAs were cloned from the skin secretion-derived cDNA libraries of *Bombina orientalis*. The nucleotide of full ORF of the cloned precursor transcripts and its translated sequences are shown in Figure 1, which contains 139 residues and encodes a novel bombinin (BHL-bombinin) and a novel bombinin H (bombinin HL). The data of the novel AMPs has been deposited in Genbank Nucleotide Sequence Database with the accession code LT615078.

The sequences of two novel AMPs were subjected to online BLAST program analysis with the NCBI on-line portal. The resulting typical primary structures were compared in Figure 2. The BHL-bombinin and bombinin HL, which exhibited as tandem mature peptides in biosynthetic precursor in Figure 1, revealed 96% and 82% sequence identity respectively, with other bombinins identified from *Bombinatoridae*. The main sequence difference was indicated in the last two or three residues in the C-terminus, where BHL-bombinin is -Ala-Asn- loss and bombinin HL is truncated of -Lys-Lys-Ile- with a typical Valine residue at 12th position from N-terminus (Figure 2).

3.2 Identification and structure characterisation of novel bombinin and bombinin H by rp-HPLC and MS/MS fragmentation

HPLC fractions with molecular masses coincident with predicted from molecular cloning for BHL-bombinin and bombinin HL were identified (Figure 3) following detected by the ion trap of the LCQ Fleet mass spectrometer with further tested by MS/MS fragmentation sequencing of doubly-charged ions derived from frog skin secretions (Figure 4 and 5).

3.3 Circular dichroism (CD) spectra and bioinformatic analysis

The secondary structures of synthetic replicates of AMPs were investigated in 10 mM of ammonium acetate (pH 7.0, mimicking aqueous environment) and 50% trifluoroethanol (TFE, mimicking the hydrophobic environment of the microbial membrane) by CD spectroscopy. As shown in Figure S2, all the peptides displayed random coil conformations in the aqueous environment. However, the spectrums of peptides were characteristic of α-helix conformations in the presence of 50% TFE, as indicated by the presence of double negative dichroic bands at approximately 208 and 222 nm. The

web server K2D3 calculation revealed that the helical content for BHL-bombinin is 87.59%, and for bombinin HD and bombinin HL are 77.73 % in 50 % TFE solution.

The physiochemical parameters of novel AMPs are listed in Table 1, which not only provides evidence of the possible interactions between peptides and bacterial membrane but also gives more information on their synergistic mechanisms. The molecular masses of synthetic peptides were determined by MALDI-TOF (Figure S1). Physiochemical parameters including charge, hydrophobic moment and hydrophobicity were determined using the Heliquest server [21]. The hydrophobic moment (μH) was determined by Eisenberg's scale with a full window in Heliquest server. BHL-bombinin elicits higher cationic but much less hydrophobic properties than bombinin HL, which exhibit a highly structural and physiochemical differences between the two co-encoded AMPs. These findings suggest that these two peptides may possess distinguishing roles in the interaction with micro-organisms and exhibit potent antibacterial activities synergistically.

3.4 Antimicrobial and haemolytic activities

The antimicrobial effects of synthetic AMPs on the growth of the tested microorganisms, and the biofilm eradication effects on *S. aureus* are illustrated in table 2. The BHL-bombinin exhibited stronger antimicrobial activities on Gram-positive bacteria (MIC/MBC: 4mg/l/16mg/l) and yeast (MIC/MBC: 4mg/l/16mg/l) than Gram-negative bacteria (MIC/MBC: 16-64mg/l/64-128mg/l). In addition, BHL-bombinin was found to possess a relatively low level of haemolytic activity (0–12.6%) at the MIC determined against *S. aureus* and *C. albicans* (Figure S3). Interestingly, BHL-bombinin displayed potent inhibitory effects (MIC: 4-16mg/l) towards MRSA and biofilm. By contrast, the MIC values for bombinin HL and bombinin HD against *S. aureus* were 256mg/l and 128mg/l respectively with undetected MBC, which were significantly less effective compared to BHL-bombinin. The selectivity indices (SIs), which represent the degree of antibacterial selectivity, are showed in Table 2, higher SI value reflecting a better selectivity towards microbial over mammalian membranes [22]. As indicated, the BHL-bombinin had a higher SI compared to bombinin HL and bombinin HD, which is in agreement with previous studies that high level of hydrophobicity may decrease the antimicrobial selectivity of α-helical peptides [23]. Additionally, compared with the melittin peptide, all the AMPs

investigated in this study exhibited 32 to 128 times higher SI values, which emphasises that amphibian-derived AMPs are potential research targets for therapeutic alternatives to current antibiotics. The time-killing curves demonstrated the faster cell-killing effects of BHL-bombinin compared with the ampicillin, while the kill rates of bombinin HL and bombinin HD were relatively low (Figure S4).

The combined administration of BHL-bombinin with either bombinin HL or bombinin HD revealed a synergistic antimicrobial effect against *S. aureus* (FICI 0.375). In addition, BHL-bombinin showed additive property with classic antibiotics ampicillin (FICI 0.75), while the novel bombinin H, either D- or L-amino isoforms, displayed synergistic activities with β-lactam and ampicillin (FICI 0.5). The synergistic effects were further confirmed by the outcomes of time-killing assays. Figure 6 (a) (b) exhibits that the isolated *S. aureus* had a 6.16(±0.76) log10 decrease in cfu/ml at 24 h when incubated with BHL-bombinin (0.75 mg/l) and bombinin HL (48 mg/l), compared to the single peptide effect. The time-killing value was 5.83(±0.67) log10 for combined effects of BHL-bombinin (0.75mg/l) and bombinin HD (24 mg/l). The synergistic effects were also observed when co-administrated bombinin HL (64 mg/l) with ampicillin (0.016 mg/l), or bombinin HD (32 mg/l) with ampicillin (0.016 mg/l), which demonstrated a 7.51 (±0.97) log10 and 6.57 (±0.77) log10 decrease in CFU/ml at 24 h respectively.

3.5 Cytotoxicity assessment of novel bombinin, bombinin H and their synergistic effect on HMECs (human microvessel endothelial cells)

The anti-proliferative effect data obtained from MTT cell viability assays of each peptide on HMEC-1 cells are represented in Figure 7(a)(b), and their IC50 values are calculated. All the AMPs tested in this study exhibited low cytotoxicity with cell viabilities exceeding 90% up to the concentration 10^{-5}M against HMEC-1. For BHL-bombinin, at MIC concentrations (1.6–26.2 μM), 83.5–100.0% HMEC-1 cells remained viable. For bombinin HL and bombinin HD, they displayed relatively lower selectivity and higher cytotoxicity on HMEC-1 cells compared with BHL-bombinin. To identify the possible synergistic cytotoxicity between BHL-Bombinin and bombinin HL or bombinin HD, the cells were cultured with combinations of these two peptides at different doses but in a constant ratio (BHL-

bombinin to bombinin HL or bombinin HD: 5 μM to 10 μM, 10 μM to 20 μM, and 20 μM to 40 μM, respectively) for 24 h. The combination of 20 μM BHL-bombinin with 40 μM bombinin HL inhibited cell growth of 52.21%, compared with mono-administration of BHL-bombinin (43.93%) or bombinin HL (9.63%), indicating an additive effect (CI = 1.03; Q = 1.06). The values for combination of BHL-bombinin and bombinin HD were CI = 0.98; Q = 1.10. The results revealed that the synergistic relationship was abolished with $0.85 < Q < 1.15$ and $CI \geqslant 1$ with regards to their cytotoxicity on normal mammalian cells.

4. Discussion

Different from the well-studied bioactive peptides from the amphibian *Pipidae*, *Hylidae*, *Ranidae*, and *Pseudidae* families, skin secretions from *Bombina* species, remain to be investigated fully and may yield valuable promotion for drug development. The best-known constituent identified from *Bombina* skin secretions is bombesin, which led to the subsequent identification of the mammalian homologues, gastrin-releasing peptide (GRP) and neuromedin B (NMB) as neuropeptides [24]. Among all the molecules secreted from *Bombina* species, no counterparts of the novel BHL-bombinin and bombinin HL, which are encoded by single coding region precursor, have been identified in other amphibian genera or in mammals [25].

The present study describes the molecular cloning, primary structure identification, chemical synthesis, and bioactive examinations of two tandem-coded novel bombinin peptides. Since the D-isomer exists in some of the bombinin H-type molecular at the 2^{nd} position of their sequences, the analogue bombinin HD was designed by substituting the L-leucine at such position. CD studies revealed that the helical content of BHL-bombinin was only approximately 10% higher than bombinin HL and bombinin HD, while the hydrophobicity of BHL-bombinin is significant lower than that bombinin HL and bombinin HD. Therefore, all the tested AMPs in this study were found to adapt an amphipathic α-helical conformation in a membrane mimetic environment, a feature that is essential for allowing AMPs to exert their bioactivities [26]. However, due to the diversities of their primary

structures and physiochemical parameters, the functional mechanisms that they employed can be significantly different.

Synthetic BHL-bombinin were found to possess potent antimicrobial activities against *S. aureus* and *C. albicans*, but relatively lower activity against *E. coli* and *P. aeruginosa*. The MBCs for all the four tested micro-organisms were approximately equal to or over 4-fold of their respect MICs. Clinically, the formation of biofilm and conventional antibiotic resistant strain MRSA are two major causes of antibiotic crisis. BHL-bombinin showed potent effects for eliminating *S. aureus* biofilm and inhibiting the growth of isolated MRSA. However, the MICs observed for wild-type bombinin HL and analogue bombinin HD, were moderately effective against *S. aureus* with undetected MBC. The antimicrobial properties of the peptides reported in this study were further compared against melittin, which is a well-studied bee venom derived AMP [15]. BHL-bombinin exhibited similar antimicrobial activity to melittin, but weaker haemolytic activity, which indicates a better selectivity. Both bombinin H peptides possessed mild antibacterial property but higher selective antimicrobial activity compared to melittin. Following on from this, the BHL-bombinin and bombinin HL are tandem-encoded in single ORF, which prompt us to speculate that their combination effect might be vital for frogs to survive in pathogens-rich environments. Of note, the combination effect between novel components and conventional antibiotics has also been proven as a promising solution to amplify the potency of antibiotics, a good example is Co-amoxiclav, which enormously enhances the Amoxicillin potency after combined use of clavulanic acid [27]. As expected, the combination interaction of BHL-bombinin with either bombinin HL or bombinin HD showed synergistic inhibition activities against *Staphylococcus aureus* (FICI 0.375). Moreover, BHL-bombinin showed additive effect with classic antibiotics ampicillin (FICI 0.75), while the bombinin HL and HD, displayed synergistic activities with β-lactam, ampicillin (FICI 0.5). The results were further confirmed by time-killing assays, that the BHL-bombinin exerted higher bactericidal rate compared with ampicillin. However, the killing rates for bombinin HL and bombinin HD were diminished. The mechanism of the positive outcomes between peptides and conventional antibiotics (ampicillin in this study) appears to be complex. The fractional inhibitory concentration (FIC) index as a measure of synergy employed in this study is the

best known and a very basic method for evaluating the inhibitory effects of paired agents comparing the sum of their effects alone. We calculate their FICI according to the protocol for investigating their synergistic relationships at preliminary level in this study [17,18]. For addressing the more complicated natural environment, the detailed concentration and structural relationship between peptides in this work needs a further and more systematic mechanism evaluation, which depends on the physiochemical parameters and their combination results of BHL-bombinin and bombinin HL, BHL-bombinin may have direct and selective membrane permeabilizing-activity, which increases the uptake of other antibacterial agents that initiate the process to interfere with intracellular targets or enhance the effect of highly hydrophobic molecules like bombinin HL. On the other hand, either bombinin HL or bombinin HD may cause degradation of the peptidoglycan by triggering the activity of bacterial murein hydrolases, which can enhance the activity of the β-lactams [28-30].

Safety evaluations via haemolytic assay demonstrated the relatively lower SIs of both bombinin HL and bombinin HD on horse erythrocytes compared with BHL-bombinin, as a consequence, when treated HMEC-1 cells with the peptides using their MIC concentrations for MTT-based viability assessment, bomhinin HL and HD exhibited higher cytotoxicity. The typical theory is that the increased hydrophobicity of the AMPs is associated with higher antimicrobial activity, but in contrast, the high hydrophobic peptides are more associated with stronger self-assembly, which can result in the formation of dimers or oligomers. This spatial character may in turn decrease their potential for passing through the target cell wall and bacterial membrane [31]. The high toxicity of BHL-bombinin in this study might be mainly due to its innate character of highly hydrophobicity. Additionally, the abolishment of combined effect of BHL-bombinin and bombinin H against HMEC-1 revealed their high functional selectivity. The application of combined antimicrobial agents, either with AMPs or with conventional antibiotics, is a prospective strategy to improve clinical therapy caused by multi-drug resistant pathogens and decrease the side-effect [32].

5. Conclusion

In this project, the novel BHL-bombinin, bombinin HL and analogue bombinin HD are reported from less-studied frog species *Bombina orientalis*. They revealed comparable antimicrobial property individually and enhanced synergistic effect and selectivity jointly, all these inherent and robust characteristics hold significant potential to alleviate the current antibiotics crisis.

Author contribution

Jie Xiang, Yuxin Wu and Tianbao Chen conceived and designed the experiments. Jie Xiang performed the experiments. Jie Xiang, Yuxin Wu and Mei Zhou analysed the data. Chris Shaw and Lei Wang contributed reagents/materials/analysis tools. Jie Xiang and Yuxin Wu wrote the paper. Yuxin Wu and Tianbao Chen edited the paper.

Declaration of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure legends and Tables

Figure 1 The nucleotide sequence and open-reading frame amino acid sequence of full length preprobombinin and preprobombinin H peptides encoding cDNA from the oriental fire-bellied toad, *Bombina orientalis*. The putative signal peptide was double-underlined. The mature peptide was single-underlined for bombinin and dash-underlined for bombinin H. The stop codon was indicated by an asterisk.

Figure 2 Alignment of the primary structure of the novel peptides BHL-Bombinin (a) and bombinin HL (b) with other bombinin family peptides. An * (asterisk) indicates positions that have a single, fully conserved residue. Substitutions are highlighted in grey. D-amino acids are boldfaced and italic. Gap residues are represented by dashes. [a] Sequences deduced from BLP-3 and BLP-7 genes, GH means gene-derived bombinin H-like peptide.

Figure 3 Region of reverse phase HPLC chromatogram of *Bombina orientalis* skin secretion with arrow indicating the retention times of the novel BHL-Bombinin (1) and Bombinin HL (2). The detection wavelength was 214 nm with a flow rate of 1 ml/min in 240 min.

Figure 4 Thermoquest LCQ™ fragment scan spectrum derived from ions corresponding to BHL-Bombinin (a) and electrospray ion-trap MS/MS fragmentation dataset (b). Expected single- and double-charged b- ions and y-ions arising from MS/MS fragmentation were predicted using the MS Product programme through Protein Prospector on-line. Truly observed ions are indicated in bold typeface and underlined.

Figure 5 Thermoquest LCQ™ fragment scan spectrum derived from ions corresponding to Bombinin HL (a) and electrospray ion-trap MS/MS fragmentation dataset (b). Expected single- and double-charged b- ions and y-ions arising from MS/MS fragmentation were predicted using the MS Product programme through Protein Prospector on-line. Truly observed ions are indicated in bold typeface and underlined.

Figure 6 Time-killing curves for combinational treatment of peptides and antimicrobial agents against *S. aureus*. Control (red circle/line), bombinin HL and bombinin HD (green square/line), ampicillin (brown triangle/line), BHL-Bombinin (purple triangle/line), combination pairs (black inverted triangle/line), The detection limit is indicated as a dashed line. The graphs were derived from values of three independent trials.

Figure 7 Cytotoxicity assessment of AMPs. Dose-dependent antiproliferative effects of BHL-Bombinin (a), bombinin HL and bombinin HD (b) against HMEC-1 after 24 h of incubation (V, N and P represent vehicle control, negative control and positive control respectively). The levels of significance are: * $p < 0.05$; ** $p <$

0.01; *** $p < 0.001$ versus vehicle. The growth inhibition rate graphs of combination effects among a series concentration between BHL-bombinin with bombinin HL(c) or bombinin HD (d) against HMEC-1 after incubation for 24 h. (e) Combination index Q of the combination treatment of synergistic pairs, where $Q < 0.85$, $Q > 1.15$ and $0.85 < Q < 1.15$ represent antagonism, synergy, and additive effect, respectively. (f) CI-effect plots were generated using CalcuSyn software. The points represent CI values for the combinations 5, 10, 20 μM BHL-bombinin with 10, 20, 40 μM bombinin HL or bombinin HD in a constant ratio against HMEC-1.

Table 1 Physico-chemical characteristics of the novel AMPs BHL-Bombinin and Bombinin HL.

Table 2 The susceptibility of novel AMPs and melittin peptide against microbial strains, bacterial biofilm and their selectivity indices (SIs) against *S. aureus*. [a] NA: not active; no inhibition or bactericidal activity was observed using peptide concentrations up to and including 512mg/L. [b] SI (selectivity index) defined as the ratio of HC_{50} to MIC against *S. aureus* value (HC_{50}/MIC). When no or mild haemolysis was observed at the highest concentration employed (512 mg/l), a value of 1024 mg/l was used for calculation.

Table 3 The combinational effects of the novel AMPs with ampicillin against *S. aureus* using checkerboard titration method. [a] The MIC values of Ampicillin is 0.0625 mg/l against *S. aureus*.

Figure 1

```
          M  N  F   K  Y  I  V   A  V  S   F  L  I   A  S  A  Y
      1   ATGAATTTTA AGTACATAGT TGCAGTGTCC TTTTTAATAG CATCTGCATA
          TACTTAAAAT TCATGTATCA ACGTCACAGG AAAAATTATC GTAGACGTAT
            A  R  S   V  Q  N   D  E  Q  S   L  S  Q   R  D  V
     51   TGCACGAAGT GTACAGAATG ATGAACAGTC TCTGAGTCAG AGGGATGTTT
          ACGTGCTTCA CATGTCTTAC TACTTGTCAG AGACTCAGTC TCCCTACAAA
          L  E  E  E   S  L  R   E  I  R   G  I  G  G   A  L  L
    101   TAGAAGAAGA ATCACTGAGG GAAATCAGAG GTATAGGAGG AGCCCTCCTA
          ATCTTCTTCT TAGTGACTCC CTTTAGTCTC CATATCCTCC TCGGGAGGAT
           S  F  G   K  S  A  L   K  G  L   A  K  G   L  A  E  H
    151   AGTTTTGGTA AATCAGCTTT AAAAGGCTTG GCTAAAGGAT TGGCTGAGCA
          TCAAAACCAT TTAGTCGAAA TTTTCCGAAC CGATTTCCTA ACCGACTCGT
            F  G  K   R  T  A   E  E  H  E   V  M  K   R  L  E
    201   TTTTGGGAAG AGAACAGCTG AAGAACATGA AGTGATGAAA AGACTGGAAG
          AAAACCCTTC TCTTGTCGAC TTCTTGTACT TCACTACTTT TCTGACCTTC
          A  R  M  R   D  L  D   S  L  D   Y  P  E  E   A  S  E
    251   CCAGAATGCG TGATCTAGAT TCCTTGGATT ATCCAGAGGA AGCTTCTGAA
          GGTCTTACGC ACTAGATCTA AGGAACCTAA TAGGTCTCCT TCGAAGACTT
           R  E  T   R  S  F  N   Q  E  E   I  A  N   L  F  T  K
    301   AGGGAAACCA GAAGCTTCAA TCAAGAGGAG ATTGCAAATC TTTTTACTAA
          TCCCTTTGGT CTTCGAAGTT AGTTCTCCTC TAACGTTTAG AAAAATGATT
            K  E  K   R  L  L   G  P  V  L   G  L  V   S  N  V
    351   AAAAGAGAAA CGCCTTTTGG GGCCAGTATT AGGTTTGGTT TCTAATGTAC
          TTTTCTCTTT GCGGAAAACC CCGGTCATAA TCCAAACCAA AGATTACATG
          L  G  G  L   L  G  *
    401   TTGGAGGTTT ACTTGGATAA TTATAGCCAG TAAAACTTTG CTTTCATTAA
          AACCTCCAAA TGAACCTATT AATATCGGTC ATTTTGAAAC GAAAGTAATT
    451   GTCAGTAAAC TTGCTTCATT ATGTTGTAAA TGAGCTATCA GATACATATA
          CAGTCATTTG AACGAAGTAA TACAACATTT ACTCGATAGT CTATGTATAT
    501   TAAGCATAGA TAAACACACA CAAAGTATTA ACAACTGCTG TCTGTACTCT
          ATTCGTATCT ATTTGTGTGT GTTTCATAAT TGTTGACGAC AGACATGAGA
    551   GCTTTAATAA AATCGATGAC AAAAAAAAAA AAAAAAAA
           CGAAATTATT TTAGCTACTG TTTTTTTTTT TTTTTTTT
```

Figure 2

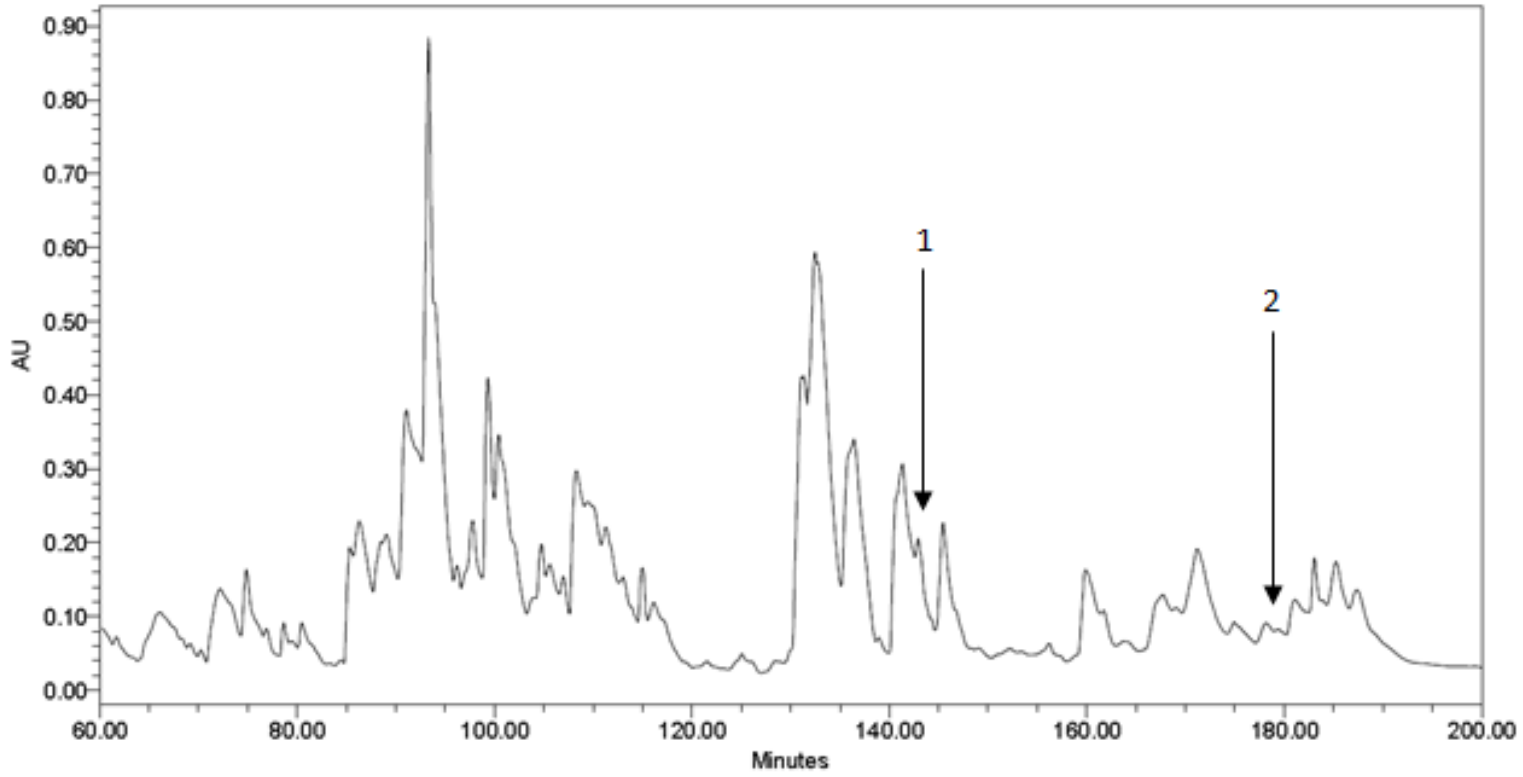
```
BHL-Bombinin                        GIGGALLSFGKSALKGLAKGLAEHF---NH2
Bombinin-like peptides BLP-3[a]     GIGAAILSAGKSALKGLAKGLAEHF---NH2
Bombinin-like peptides BLP-7[a]     GIGGALLSAGKSALKGLAKGLAEHFAN-NH2
FPA-bombinin-BO                     GIGGALLSAGKAALKGLAKGLAEHFAN-NH2
FPA-bombinin-BV                     GIGGALLNVGKVALKGLAKGLAEHFAN-NH2
Maximin-4                           GIGGVLLSAGKAALKGLAKVLAEKYAN-NH2
                                    ***  **  ** ***********
```

(a)

```
Bombinin HL                          LLGPVLGLVSNVLGGLL----NH2
Bombinin HVD                         LLGPVLGLVSNVLGGLL----NH2
Bombinin H6                          ILGPILGLVSNALGGLL----NH2
Bombinin H7                          ILLGPILGLVSNALGGLL----NH2
Bombinin H2                          IIGPVLGLVGSALGGLLKKI-NH2
Bombinin H4                          IIGPVLGLVGSALGGLLKKI-NH2
Bombinin GH-1L[a]                    IIGPVLGLVGKPLESLLE---NH2
Bombinin GH-1D[a]                    IIGPVLGLVGKPLESLLE---NH2
                                       ** ****   *  **
```

546 (b)

Figure 3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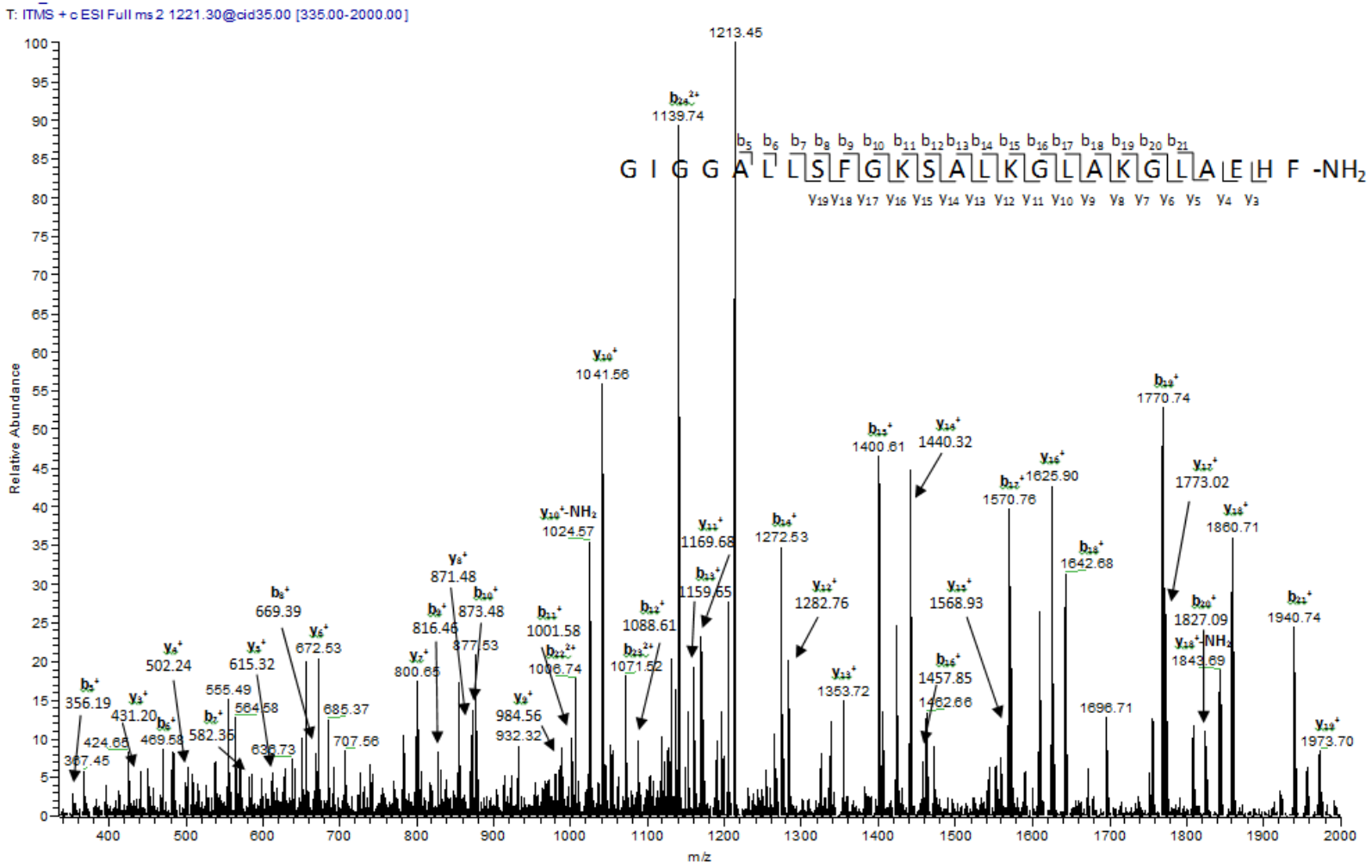
100.00
120.00
140.00
160.00
180.00
200.00
Minutes

552 **Figure 4**



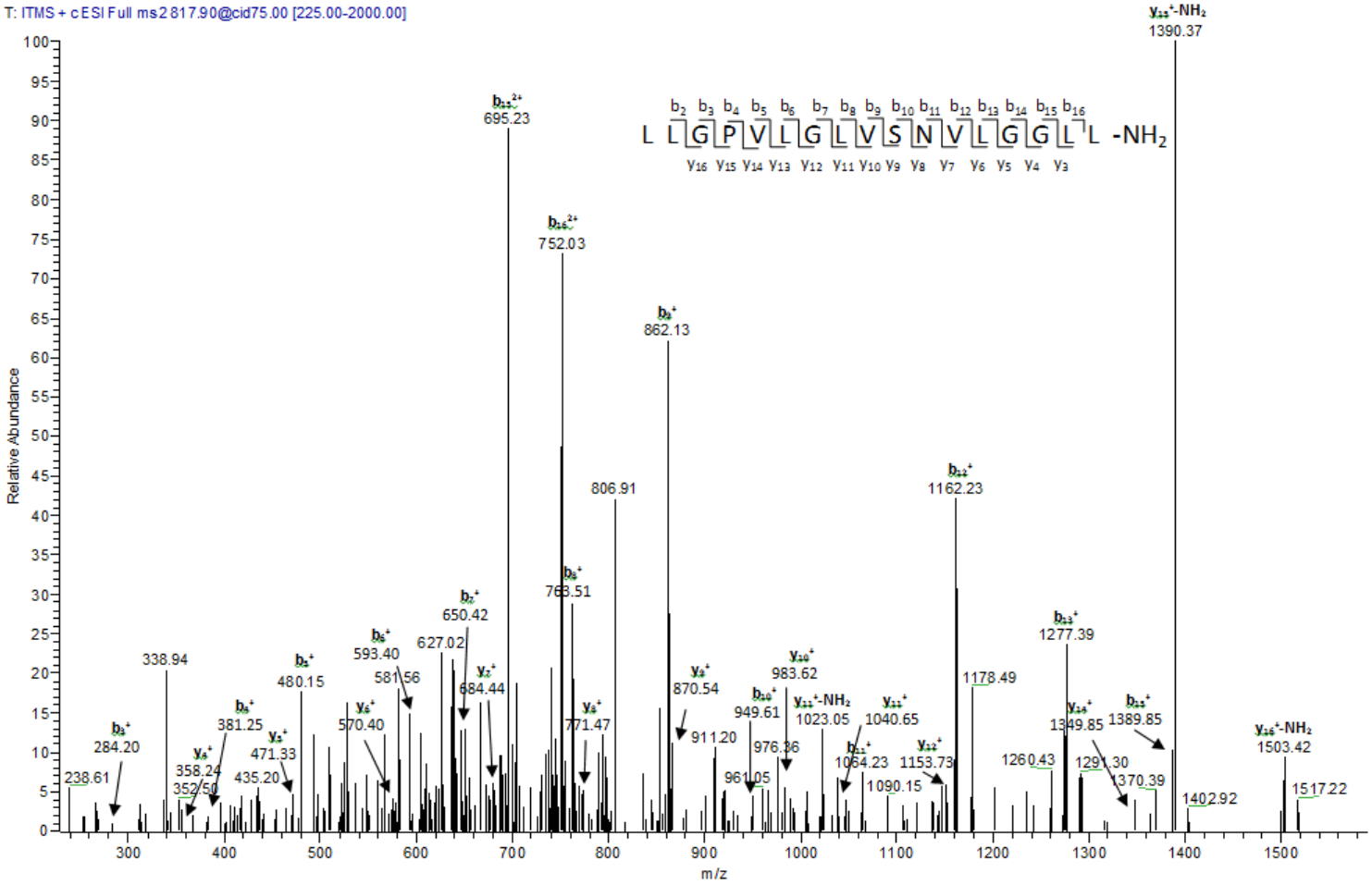
553

554 (a)

#1	b(1+)	b(2+)	Seq.	y(1+)	y(2+)	#2
1	58.03	29.52	G			**25**
2	171.11	86.06	I	2384.38	**1192.69**	**24**
3	228.13	114.57	G	2271.30	**1136.15**	**23**
4	285.16	143.08	G	2214.28	**1107.64**	**22**
5	**356.19**	178.60	A	2157.25	**1079.13**	**21**
6	**469.28**	235.14	L	2086.22	**1043.61**	**20**
7	**582.36**	291.68	L	**1973.13**	**987.07**	**19**
8	**669.39**	335.20	S	**1860.05**	**930.53**	**18**
9	**816.46**	**408.73**	F	**1773.02**	**887.01**	**17**
10	**873.48**	**437.25**	G	**1625.95**	**813.48**	**16**
11	**1001.58**	**501.29**	K	**1568.93**	**784.97**	**15**
12	**1088.61**	**544.81**	S	**1440.83**	**720.92**	**14**
13	**1159.65**	**580.33**	A	**1353.80**	**677.40**	**13**
14	**1272.73**	**636.87**	L	**1282.76**	**641.89**	**12**
15	**1400.83**	**700.92**	K	**1169.68**	**585.34**	**11**
16	**1457.85**	**729.43**	G	**1041.58**	**521.30**	**10**
17	**1570.93**	**785.97**	L	**984.56**	**492.78**	**9**
18	**1641.97**	**821.49**	A	**871.48**	**436.24**	**8**
19	**1770.06**	**885.54**	K	**800.44**	**400.72**	**7**
20	**1827.09**	**914.05**	G	**672.35**	336.68	**6**
21	**1940.17**	**970.59**	L	**615.32**	308.17	**5**
22	2011.21	**1006.11**	A	**502.24**	251.62	**4**
23	2140.25	**1070.63**	E	**431.20**	216.11	**3**
24	2277.31	**1139.16**	H	302.16	151.58	**2**
25			F-Amidated	165.10	83.05	**1**

(b)

559 **Figure 5**



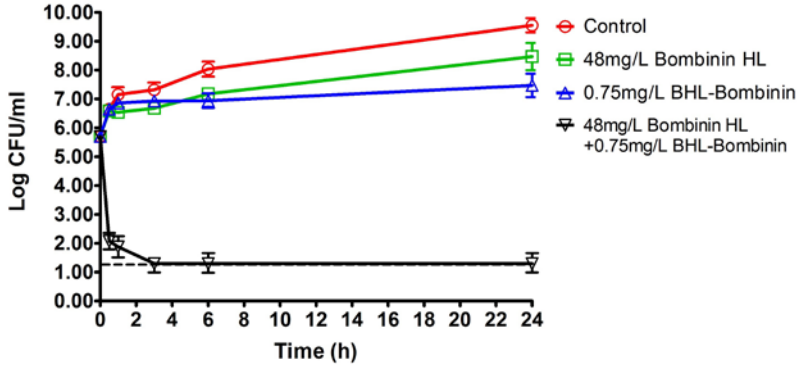
560

561 (a)

#1	b(1+)	b(2+)	Seq.	y(1+)	y(2+)	#2
1	114.09	57.55	L			**17**
2	**227.18**	114.09	L	**1519.96**	**760.48**	**16**
3	**284.20**	142.60	G	**1406.87**	**703.94**	**15**
4	**381.25**	191.13	P	**1349.85**	**675.43**	**14**
5	**480.32**	**240.66**	V	**1252.80**	**626.90**	**13**
6	**593.40**	**297.20**	L	**1153.73**	**577.37**	**12**
7	**650.42**	**325.72**	G	**1040.65**	**520.83**	**11**
8	**763.51**	**382.26**	L	**983.62**	**492.32**	**10**
9	**862.58**	**431.79**	V	**870.54**	**435.77**	**9**
10	**949.61**	**475.31**	S	**771.47**	**386.24**	**8**
11	**1063.65**	**532.33**	N	**684.44**	**342.72**	**7**
12	**1162.72**	**581.86**	V	**570.40**	**285.70**	**6**
13	**1275.80**	**638.41**	L	**471.33**	236.17	**5**
14	**1332.83**	**666.92**	G	**358.24**	179.63	**4**
15	**1389.85**	**695.43**	G	**301.22**	151.12	**3**
16	**1502.93**	**751.97**	L	244.20	122.60	**2**
17			L-Amidated	131.12	66.06	**1**

(b)

Figure 6



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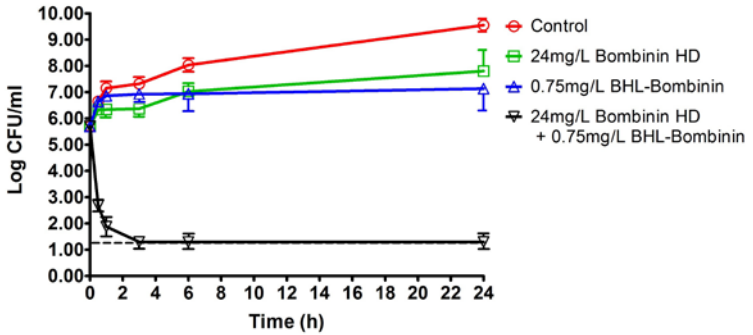
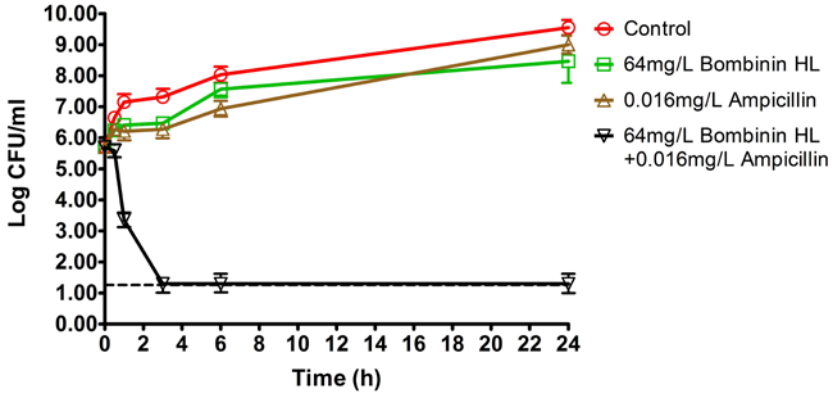
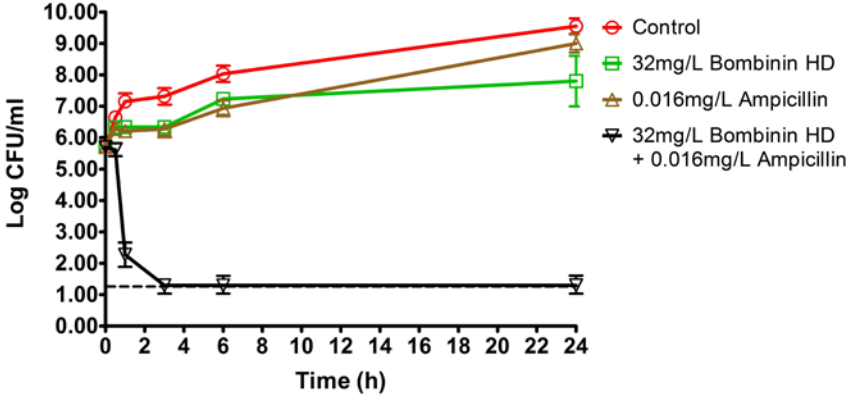


(b)



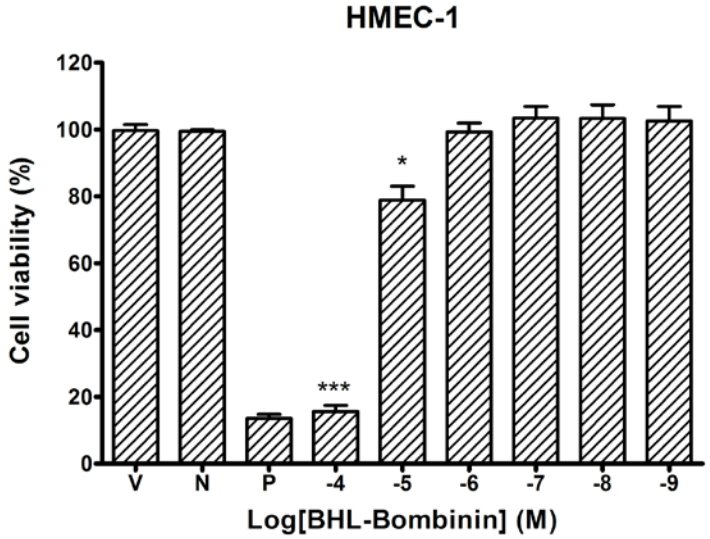
(c)



(d)

574

575 **Figure 7**



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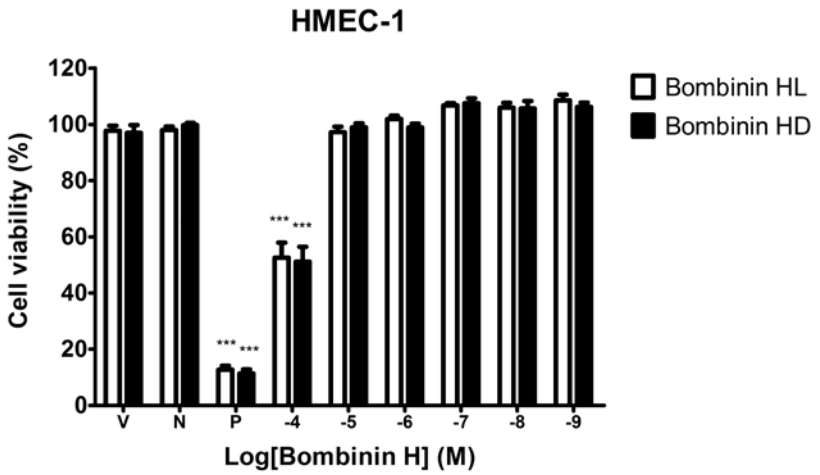


577

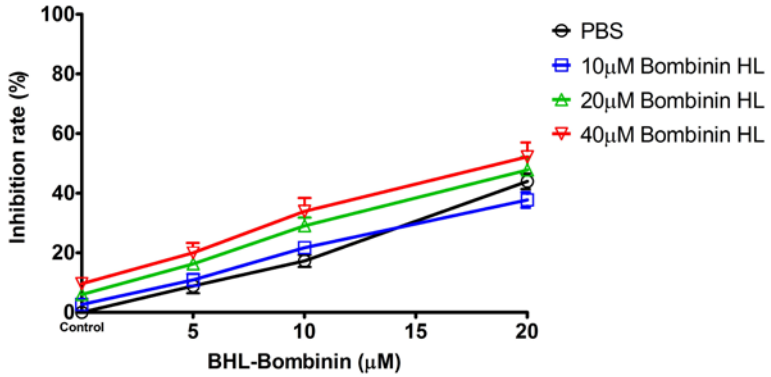
578 (a) (b)

	BHL-Bombinin	Bombinin HL	Bombinin HD
IC_{50}(μM)	94.32±0.72	103.51±0.43	104.23±0.24

579

580

581



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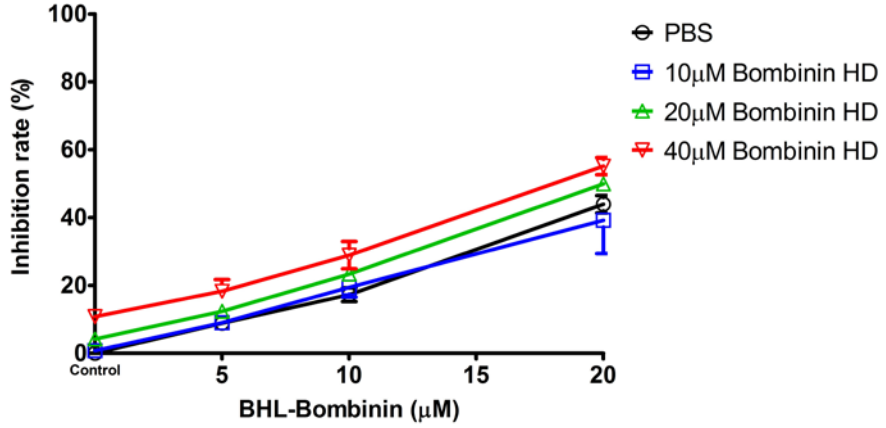
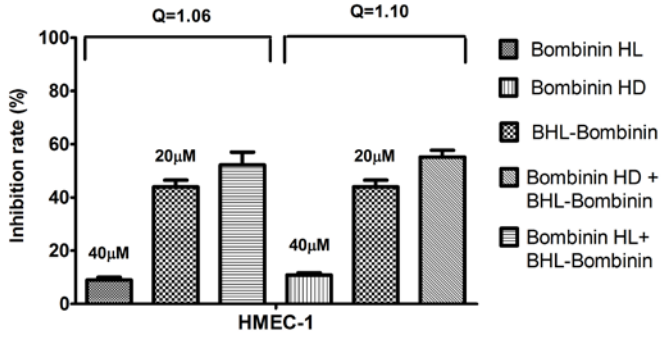


583

584 (c) (d)



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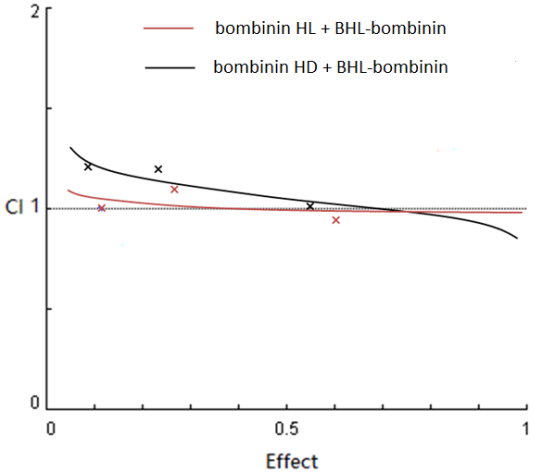


(f)

Table 1

Peptides	Theoretical Mw	Measured Mw	Hydrophobicity (H)	Hydrophobic moment (μH)	Net charge	%Helicity
BHL-Bombinin	2441.87	2441.50	0.462	0.404	+3	87.59
Bombinin HL	1633.03	1633.02	0.920	0.501	+1	77.73

Table 2

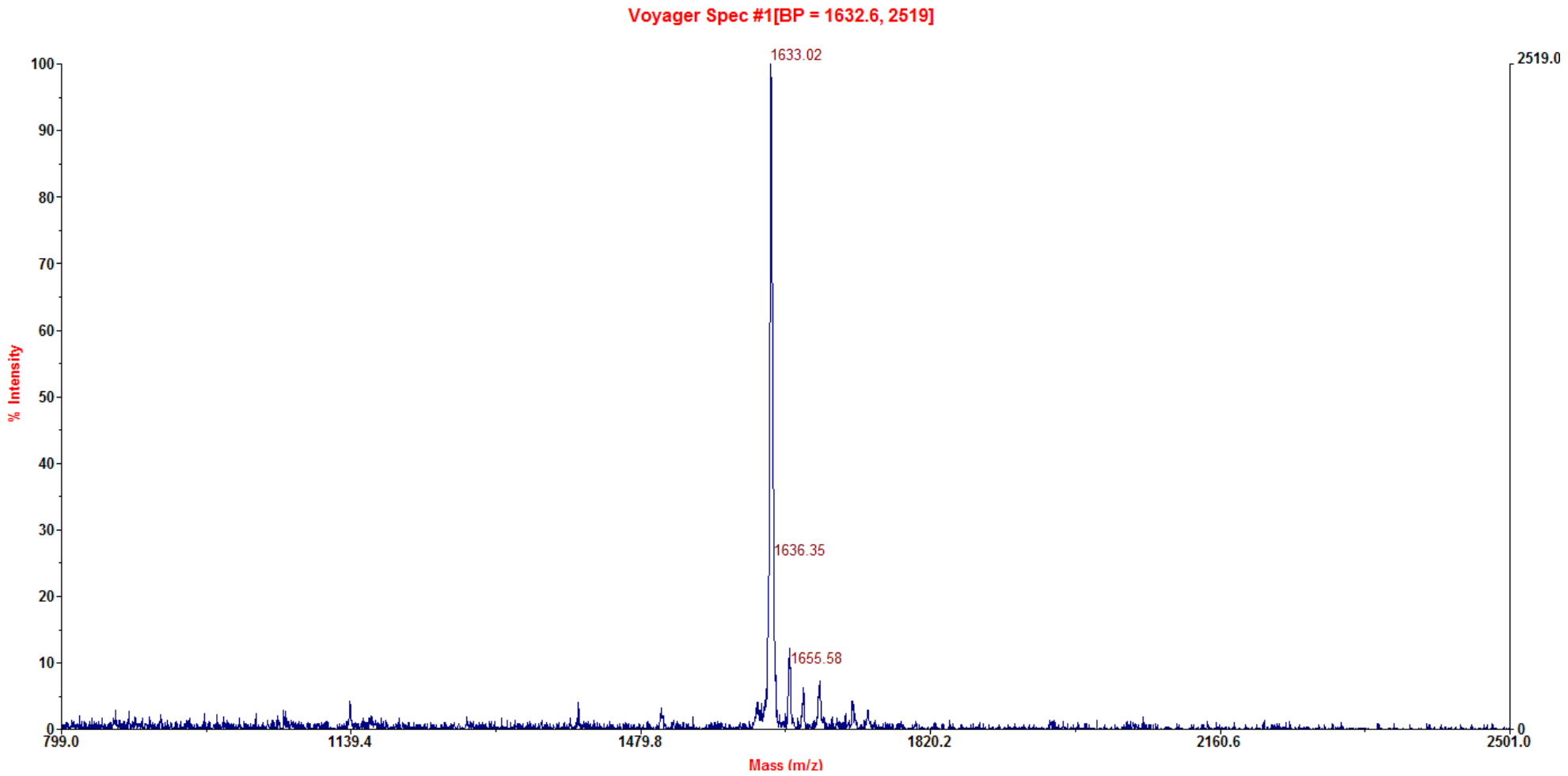
Peptides	MIC / MBC [mg/l(µM)]				MIC / MFC [mg/l(µM)]	MBEC [mg/L(µM)]	HC_{50} [mg/l(µM)]	SI[b]
	Gram-positive bacteria		Gram-negative bacteria		Fungi	*S. aureus*		
	S. aureus	*MRSA*	*E. coli*	*P. aeruginosa*	*C. albicans*			
BHL-Bombinin	4(1.6)/ 16(6.6)	16(6.6)/ 64(26.2)	16(6.6)/ 64(26.2)	64(26.2)/ 128(52.4)	4(1.6)/ 16(16.6)	4(1.6)	64(26.2)	16
Bombinin HL	256(156.8)/ NA[a]	NA/ NA	NA/ NA	NA/ NA	NA/ NA	NA	>512(313.5)	4
Bombinin HD	128(78.4)/ NA	NA/ NA	NA/ NA	NA/ NA	NA/ NA	NA	>512(313.5)	8
Melittin	8(2.8)/16(5.6)	32(11.2)/ 64(22.4)	16(5.6)/ 32(11.2)	16(5.6)/ 64(22.4)	8(2.8)/ 16(5.6)	8(2.8)	1(0.4)	0.125

Table 3

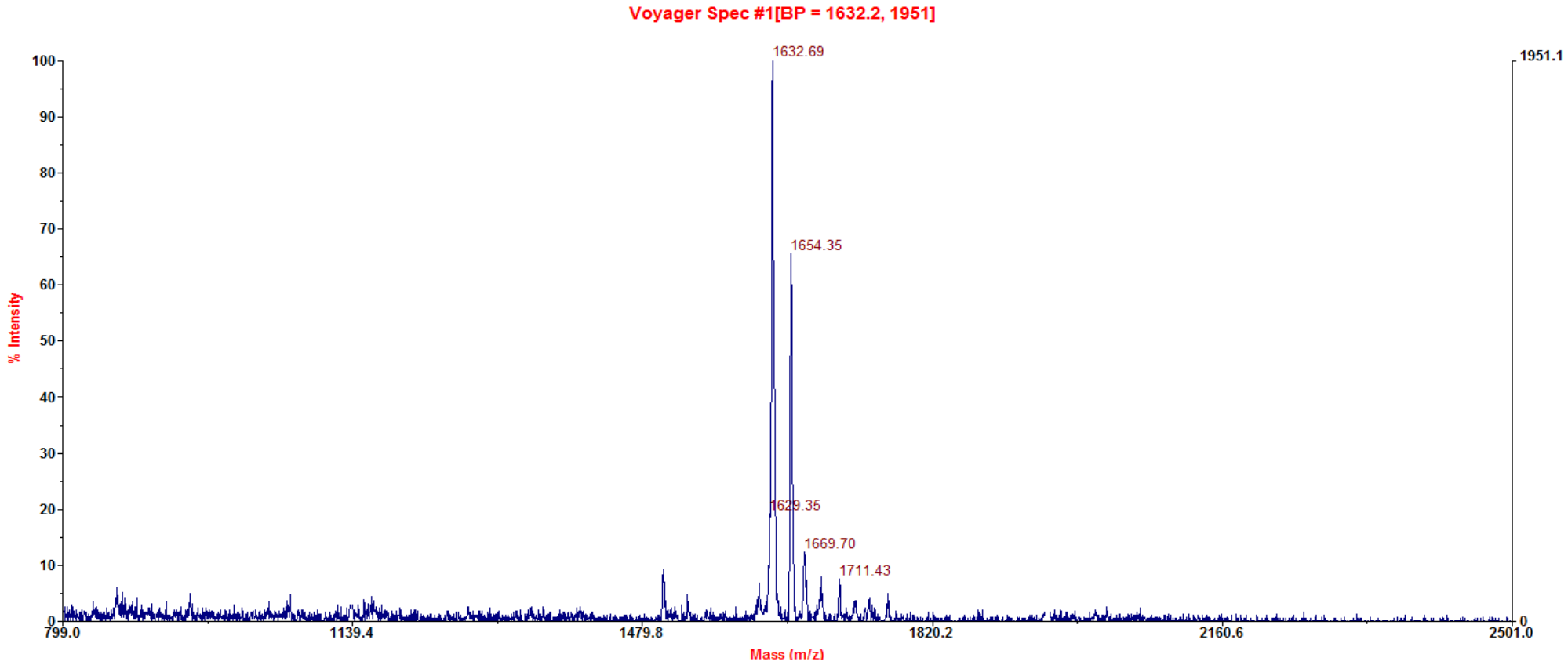
The antimicrobial treatment settings		Lowest FIC index ([A]/[B] in mg/l)	Results
A	**B**		
BHL-Bombinin	Bombinin HL	0.375(0.75/48)	Synergistic
	Bombinin HD	0.375(0.75/24)	Synergistic
	Ampicillin[a]	0.75(2/ 0.016)	Additive
Bombinin HL	Ampicillin	0.5(64/0.016)	Synergistic

Bombinin HD	Ampicillin	0.5(32/0.016)	Synergistic

Supplementary data

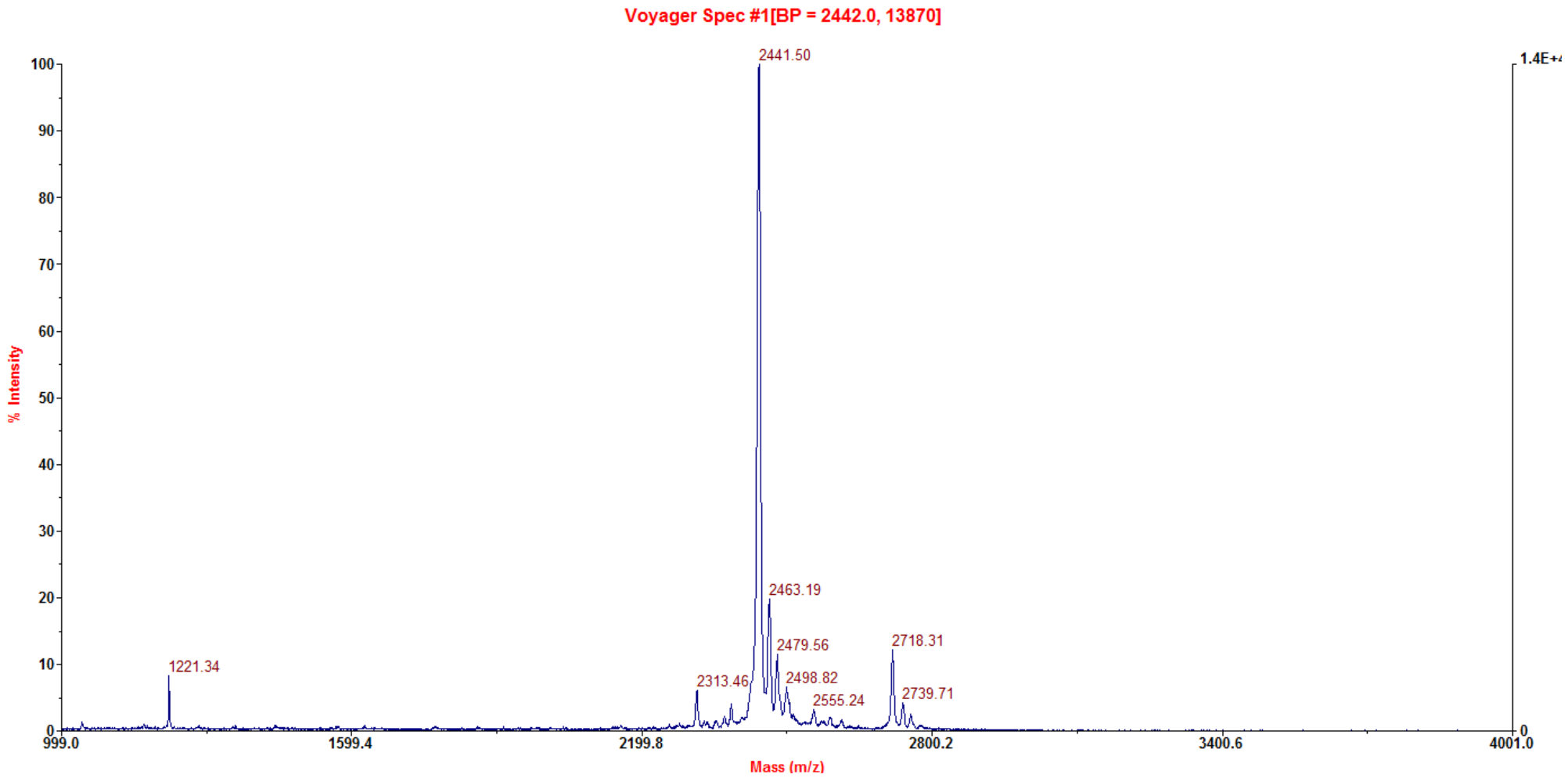


(a)


Voyager Spec #1[BP = 1632.2, 1951]
1632.69
1654.35
1629.35
1669.70
1711.43
1951.1
% Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
0
799.0
1139.4
1479.8
1820.2
2160.6
2501.0

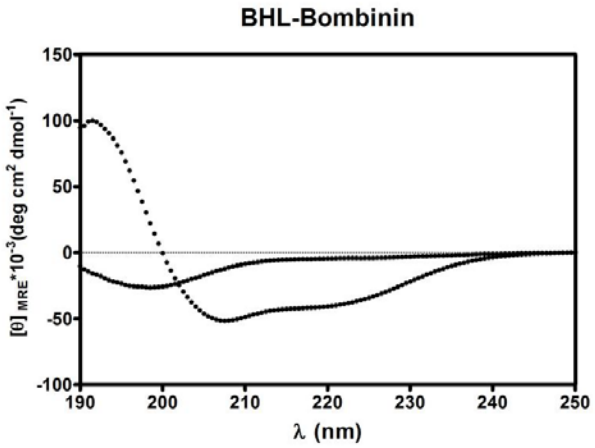
603

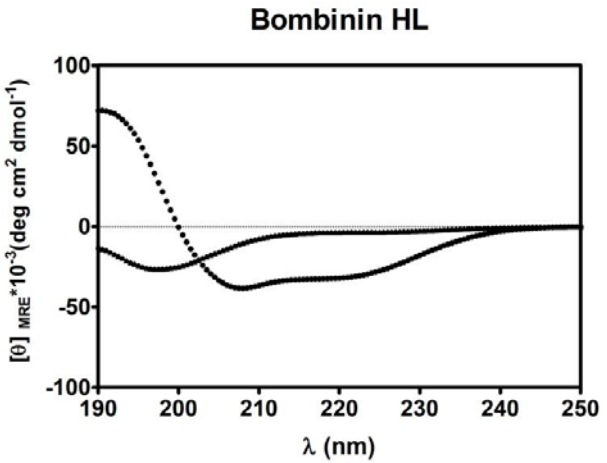
604 (b)



(c)

Figure S1 MALDI-TOF (Perceptive Biosystem, Bedford, MA, USA) mass spectrum of synthetic peptide (a) bombinin HL and (b) bombinin HD and (c) BHL-bombinin. In (b), the initial neutral molecule bombinin HD [M] and metal ion adducts ([M+Na]+: 1654.35Da and [M+K]+: 1669.70Da) were observed.





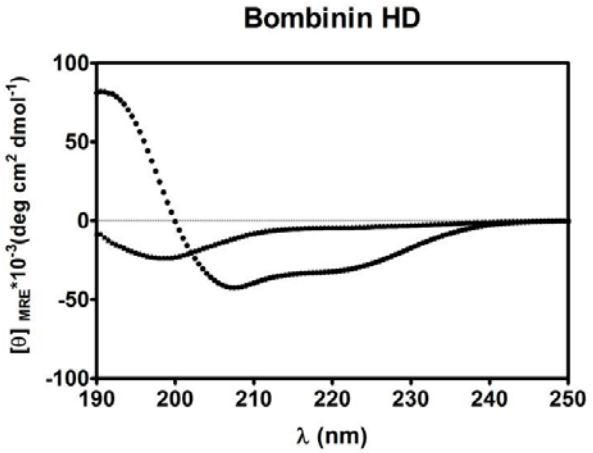


Figure S2 CD spectra of the peptides in 10 mM ammonium acetate buffer (triangles) and 50% TFE (circles). The mean residue ellipticity was plotted against wavelength. The values from three scans were calculated as average per sample. The peptide concentrations were fixed at 100 μM.

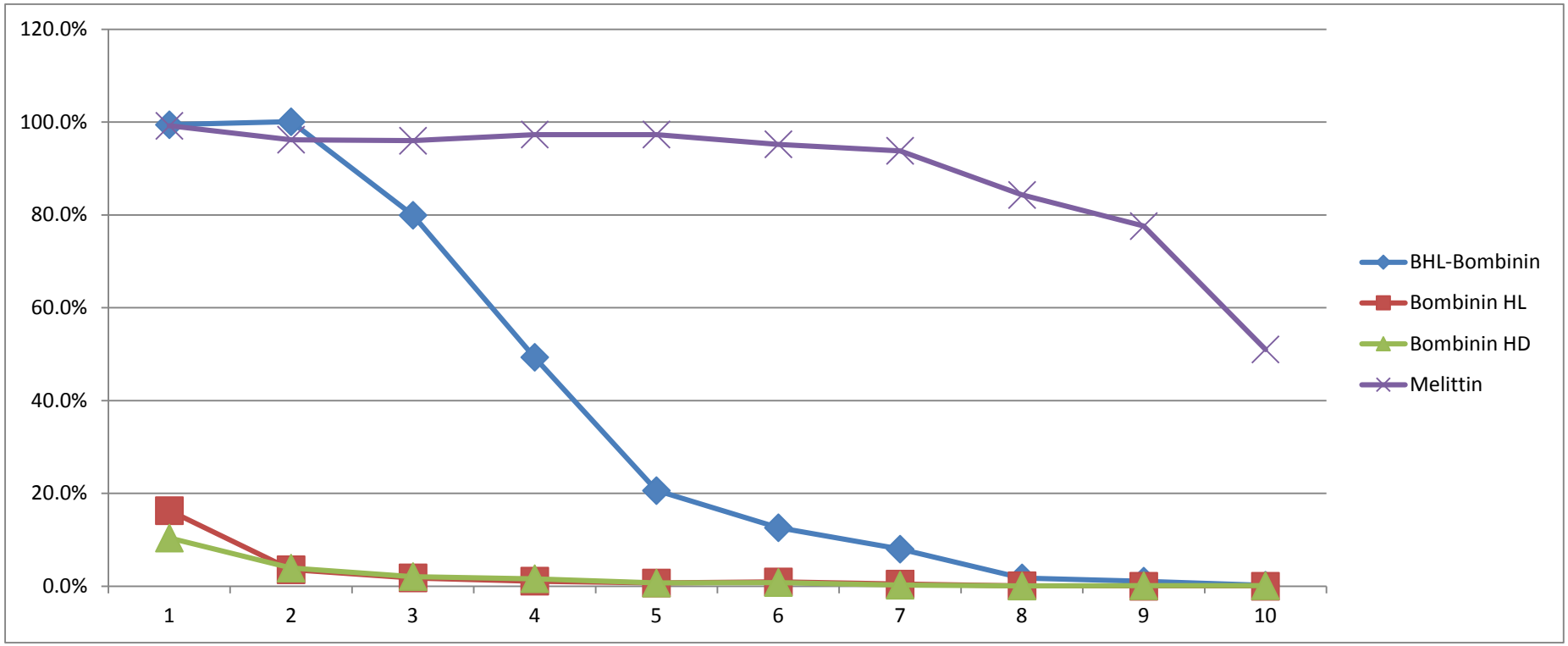
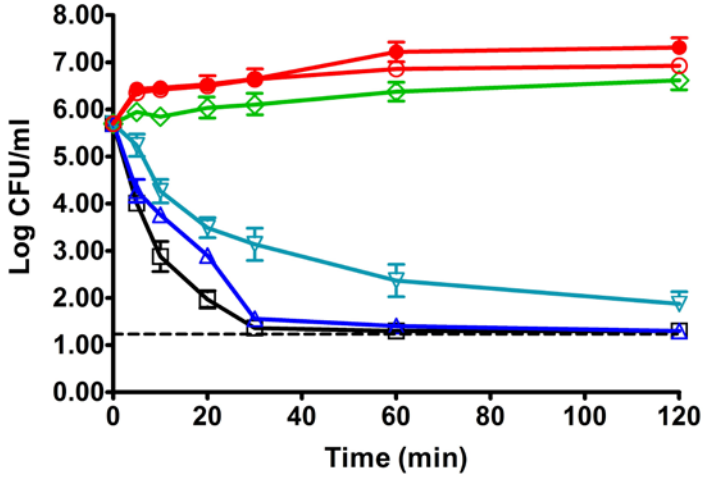
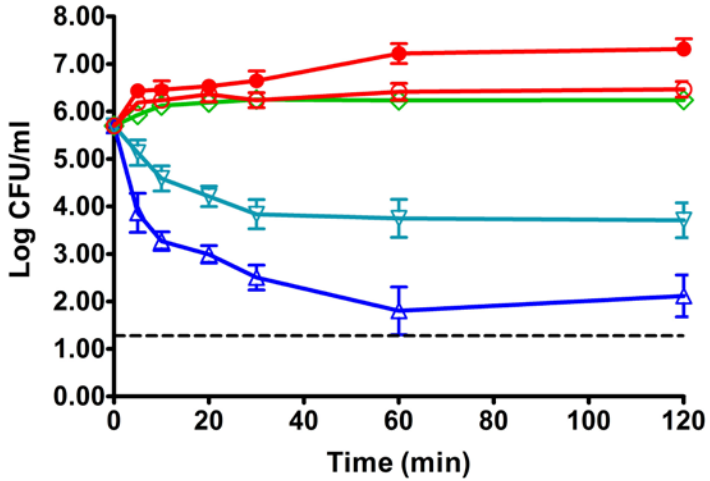


Figure S3 Haemolytic activities of BHL-bombinin (diamond), bombinin HL (square), bombinin HD (triangle) and melittin (cross) following incubation with horse erythrocytes for 2 h.

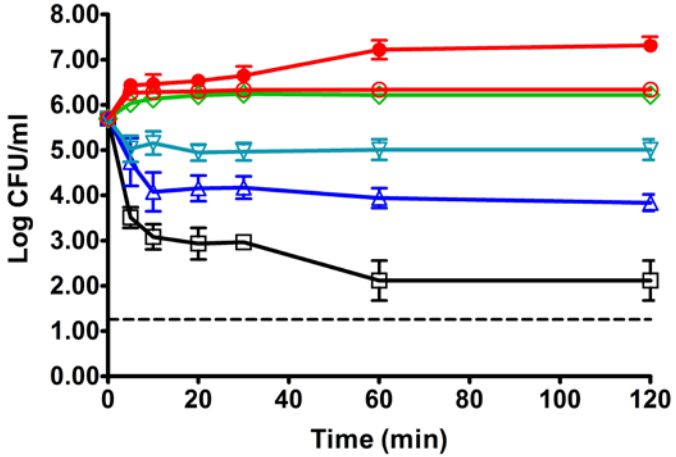


620

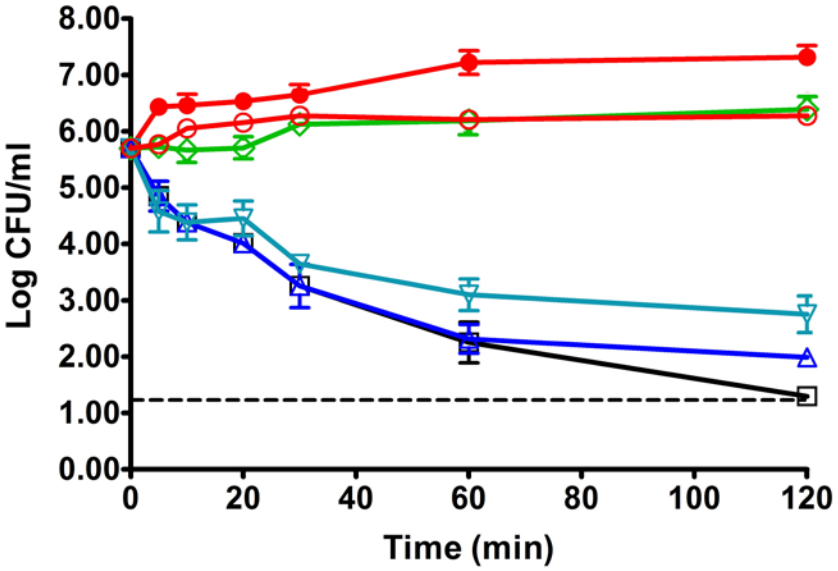


621

622 (a) (b)



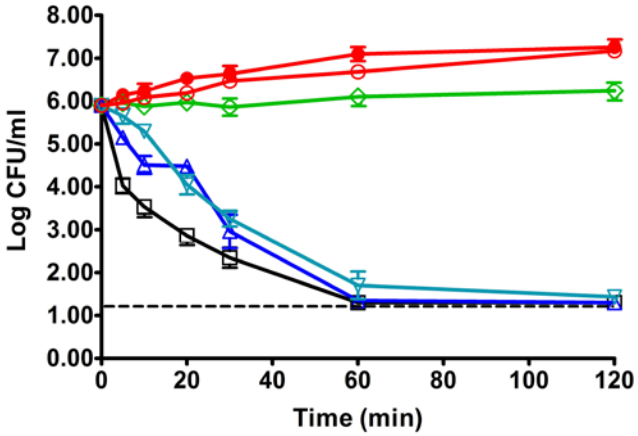
623



624

625 (c) (d)

626

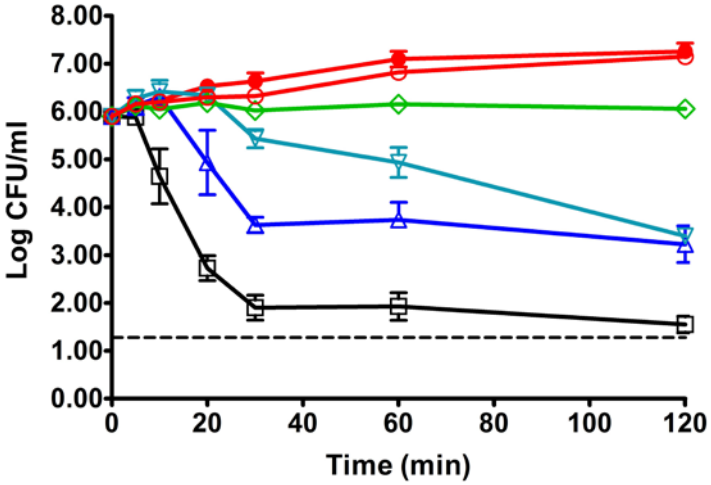


627

628 (e)

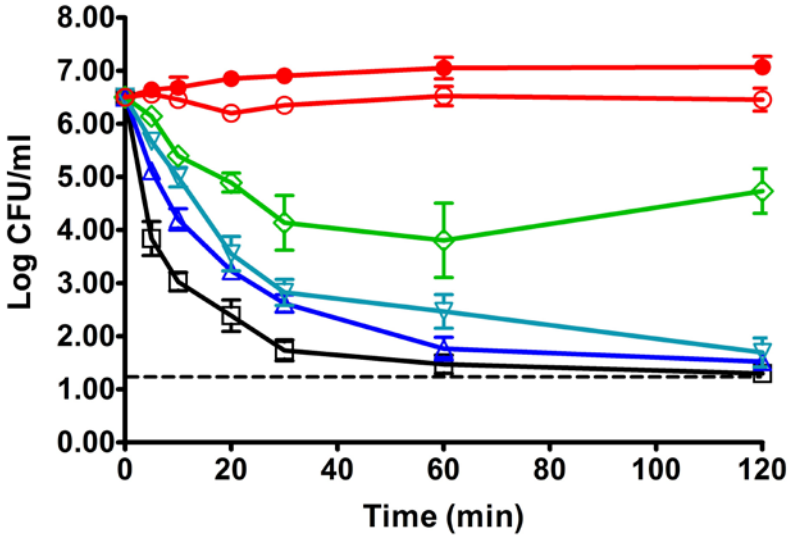
629

A



(f)

B



(g)

C

Figure S4 Time-killing curves of peptides and ampicillin at a series of concentrations: control (red filled circle), 0.25×MIC (red unfilled circle), 0.5×MIC (green diamond), 1×MIC (blue inverted triagle), 2×MIC (purple triagle) and 4×MIC (black square) against A: *S.aureus* [(a) BHL-bombinin (b) bombinin HL (c) bombinin HD (d) ampicillin]; B: *E.coli* [(e) BHL-bombinin (f) ampicillin]; C: *C.albicans* [(g) BHL-bombinin]. The detection limit was indicated as dashed line and the graphs were derived value of three independent trials.